

Activity and Stability of the Alcohol Biosensor Using *Acetobacter aceti* Biofilm on Screen-Printed Carbon Electrode

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ABSTRACT

Most of the alcohol analytical methods are robust and instrumentally expensive. An alternative of ethanol biosensor based on selected biofilm forming *Acetobacter aceti* bacteria producing alcohol oxidase was constructed on a screen-printed carbon electrode. The enzyme specifically oxidizes the ethanol and generate electrical current that then electrochemically detected and measured by cyclic voltammetry method. A scanning electron microscopic analysis indicated that the biofilm was formed firmly in the electrode. This constructed biosensor reached its optimum at biofilm formed by bacteria of 1.33×10^{10} cells/ml, temperature of 27°C, and pH 7. The enzyme kinetic had K_M and V_{max} AOX values of 3.5 mM and 125 μA respectively. The biosensor had detection and quantization limit of 0.003 and 0.009%, respectively, and a sensitivity of 57.29 μA (%)⁻¹. A linearity and relative deviation value were revealed at 0.993 and 1.95% respectively. The biosensor was relatively specific and had no interferences with methanol, sodium chloride and citric acid as the common interferences of ethanol compounds. Furthermore, the biosensor had been stably for at least 55 days. Therefore, this constructed biosensor should be developed into a prototype for a practical effective analysis.

1. Introduction

Alcohol in the form of ethanol or ethyl alcohol is widely found and used in foods and beverages. The Indonesian Ulama Council (MUI) through its decree No. 4/2003 limits the alcohol in the food and beverage products to 1%. The alcohol use as a stabilizer or food and drink coloring is limited to 0.5%. Foods and beverages containing alcohol beyond these limits are categorized as haram. Alcohol itself could be a toxic compound (Niculescu *et al.* 2002) and serve as the third largest risk factor of disease and disability in the world (Santos *et al.* 2003). According to (Rotariu *et al.* 2004) high alcohol consumption can affect the nervous system, circulatory system, and digestive system. Several methods have been developed for the determination of ethanol content such as HPLC (Yarita *et al.* 2002), GC/MS (Apers *et al.* 2003), FT-Raman spectrometry (Mendes *et al.* 2003), photometry, colorimetry (Zanon *et al.* 2007). These methods require a relatively long-time analysis,

complicated sample preparation process, expensive instrumentation, and need a trained operator (Azevedo *et al.* 2005). Therefore, a new biosensor-based electrochemical method has been developed to detect ethanol (Shkotova *et al.* 2005; Carelli *et al.* 2006; Türkarslana *et al.* 2010; Rama *et al.* 2012; Cinti *et al.* 2017). Biosensors have several advantages such as faster, simple, inexpensive, sensitive, selective techniques, and can be miniaturized (Alferov *et al.* 2011). As such, alcohol biosensors based on alcohol oxidase (Rama *et al.* 2012; Kurbanoglu and Toppare 2014; Chinnadayala *et al.* 2015) and alcohol dehydrogenase (Bilgi and Ayranci 2016; Alpat and Telefoncu 2010) have been developed. Unfortunately, their uses in biosensor construction are still limited by the tedious, time-consuming and costly enzyme purification procedures. In addition, multiple enzymes or cofactor/coenzyme are often required to generate measurable products (Wen *et al.* 2013).

In this research, a selected biofilm-forming *Acetobacter aceti* bacteria producing alcohol oxidase (AOX) was inoculated to form biofilm on a screen-printed carbon electrode (SPCE). The bacterial biofilm and its enzyme are more practically produced and manipulated than that of its pure enzyme

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immobilization (Su *et al.* 2010). The biofilm itself is relatively stable and resistant to environmental condition (Abed *et al.* 2012; Pantanella *et al.* 2013). The most important component in a biosensor is the electrode. The previous simple Carbon-based electrode has shown to be slow in collecting ions around its surface and a less stable response resulting in poor sensitivity, stability, and repetition (Xia *et al.* 2010). The objective of this study was to improve the activity and stability of ethanol biosensors by forming biofilm *A. aceti* producing alcohol oxidase on the surface of screen-printed carbon electrode.

2. Materials and Methods

2.1. *Acetobacter aceti*'s Biofilm Preparation

The selected pure colony *A. aceti* K5 was grown on *Acetobacter aceti* selective liquid medium at 30°C with a shaker speed of 90 rpm for 20 hours to reach cell density of OD₆₀₀:1,600. The bacterial cells were harvested by 10,000 rpm centrifugation at 4°C for 5 minutes. The cell pellet was wash twice and then resuspended in 50 mm phosphate buffer of pH 7. The biofilm forming assay was carried out in a microtiter plate and crystal violet staining (Merritt *et al.* 2011). The selected biofilm forming bacteria was dropped in 100 µl suspension onto the SPCE and allowed to form biofilm for 3 days. The biofilm was then observed under scanning electron microscope. The biofilm sensing acitivity was optimized using a Response Surface Method on a statistical software MINITAB with a combination of bacterial concentration variables (10⁶-10¹⁰) CFU/ml, pH (4-7), and temperature (20-40°C).

2.2. Electrochemical Measurement

Electrochemical measurement was measured by cyclic voltammetry method using eDAQ potentiostat (Ecorde 410). The measurement parameters were done as follows: *Mode Cyclic, Initial -900 mV, Final -900mV, Rate 200 mV/s, Step W 20 ms, Upper E 1,200 mV, Lower -900 mV.* Measurements were carried out with 1% ethanol solution in 50 mm phosphate buffer pH 7 as an analyte or substrate, whereas phosphate buffer was used as a blank.

2.3. Validation Methods of Biosensor

The validation was carried on analytical, kinetic, stability (Wahab 2006) and selectivity (Karthikeyan *et al.* 2012) parameters. The analytical

parameters were linearity, limit of detection, limit of quantification, sensitivity, and precision. The Linearity was determined from the calibration curve of the concentration series of 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, and 3.0% in a 50 mm phosphate buffer solution of pH 7. The sensitivity was determined based on linear regression equation of the standard curve. The limit of detection and quantification were calculated according to the linear regression equation of standard curve obtained from 6 replications of the measurements. The precision method was expressed as the percentage of relative raw deviation (% SBR). The kinetic parameters of alcohol oxidase such as K_M and V_{max} values were determined using the derivative of the Michaelis-Menten equation namely Lineweaver-Burk. The stability was determined from the measurement of 1 biosensor electrode with a certain time interval and expressed in percent. Biosensor selectivity was determined by mixed method. A solution of 1% ethanol was measured its potential, then a potential measurement of a mixed solution containing 1% ethanol solution with an interfering solution such as methanol, citric acid, and sodium chloride.

3. Results

3.1. *Acetobacter aceti*'s Biofilm Preparation

The cultured *A. aceti* had a milky white colony and short rod-shaped cells of Gram negative non spore forming bacteria (Figure 1). It is in line with the Bergey description of *Acetobacter aceti*.

The *A. aceti* formed biofilm readily on the SPCE as shown in Figure 2.

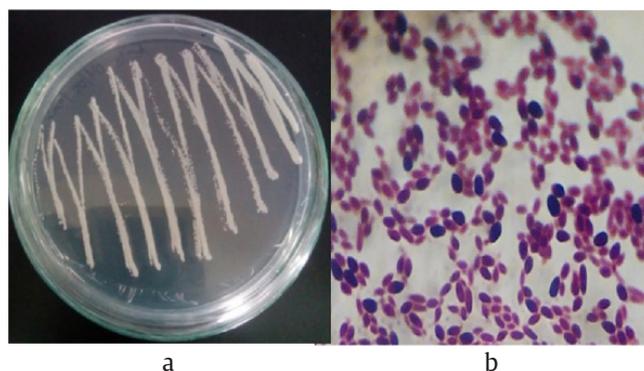


Figure 1. *A. aceti* cells (a) pure culture of *A. aceti*, (b) morphology

3.2. Electrochemical Measurement

Mechanism of detecting ethanol on SPCE biofilm *A. aceti* surface can be shown in Figure 3. The result of ethanol oxidation current measurements with SPCE biofilm was 122,429 μA (Figure 4).

3.3. Validation Methods of Biosensor

The response surface analysis revealed that the temperature significantly affected the activity as measured as electrical current of the constructed biosensor (Table 1). Relationship between two variables that affect the magnitude of the current can be seen in Figure 4. The constructed biosensor had its optimum condition at bacterial concentration of 1.33×10^{10} cells/ml, measurement temperature of 27°C and pH buffer 7 (Figure 5).

The biosensor's analytical parameters were evaluated based on linearity, sensitivity, limit detection, quantization limit, and precision parameters. The results showed that the ethanol concentration was directly proportional to the intensity of the oxidation peak current (Figure 6).

In term of selectivity, the three interfering compounds with concentrations of 0.5, 1.0, 1.5 did not interfere with the performance of electrode measurements against ethanol substrates (Table 2).

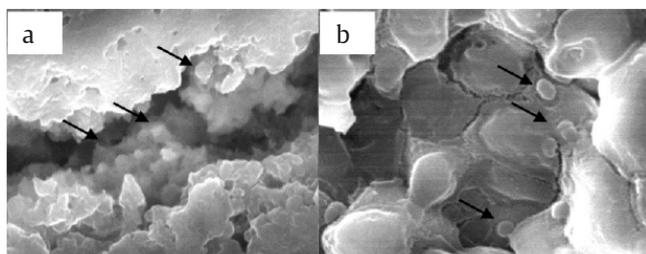


Figure 2. The scanning electron microscope images of *Acetobacter aceti* biofilm on screen printed carbon electrode. (a) before measurement and stored for 17 days, (b) after repeated measurements and stored for 17 days, observed at 10,000 magnification

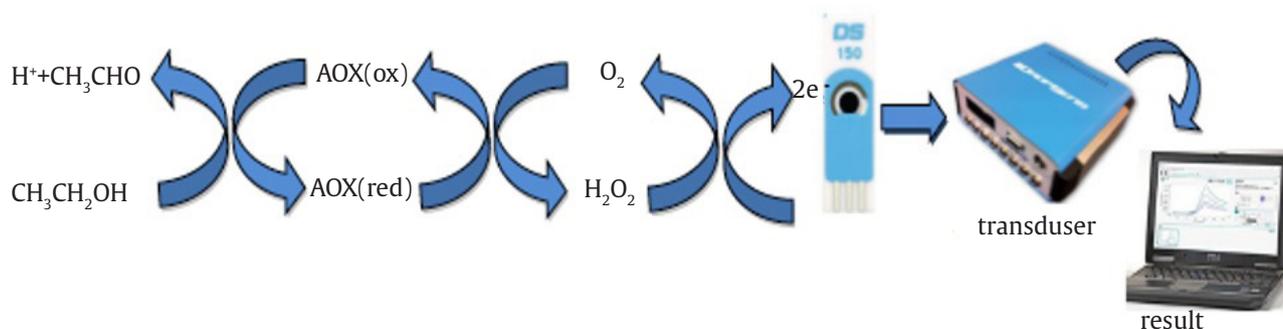


Figure 3. Mechanism of detecting ethanol on SPCE biofilm *A. aceti* surface

4. Discussion

4.1. Acetobacter aceti's Biofilm Preparation

Acetobacter aceti's biofilm preparation have been constructed. The *A. aceti* formed biofilm readily on the SPCE (Figure 2) as on the other reported surface (Karthikeyan *et al.* 2012; France 2016). The more the number of *A. aceti* cells given then the more the biofilms formed (France 2016). Initially clustered visible cells were observed that then covered by the biofilm matrix. The matrix protect and maintain the embedded living bacterial cells for a long period of time. Bacteria that have formed biofilms will be more resistant to antimicrobials than planktonic or non-biofilm bacteria (Pantanella *et al.* 2013).

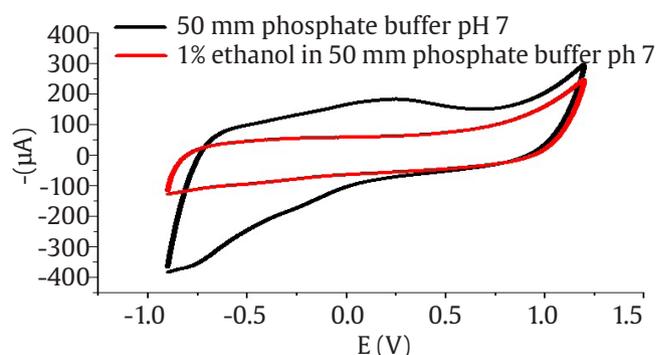


Figure 4. Cyclic voltammogram of ethanol 1% in 50 mm phosphate buffer pH 7

Table 1. Analysis result of the effect of variables towards current

Factor	Coefficient	p value
Constants	-971.833	0.001
pH buffer	123.162	0.084
Bacterial concentration	-0.594	0.991
Temperature measurement	50.144	0.000
R-Sq = 93.29%; R-Sq(pred) = 51.23%; R-Sq(adj) = 87.26%		

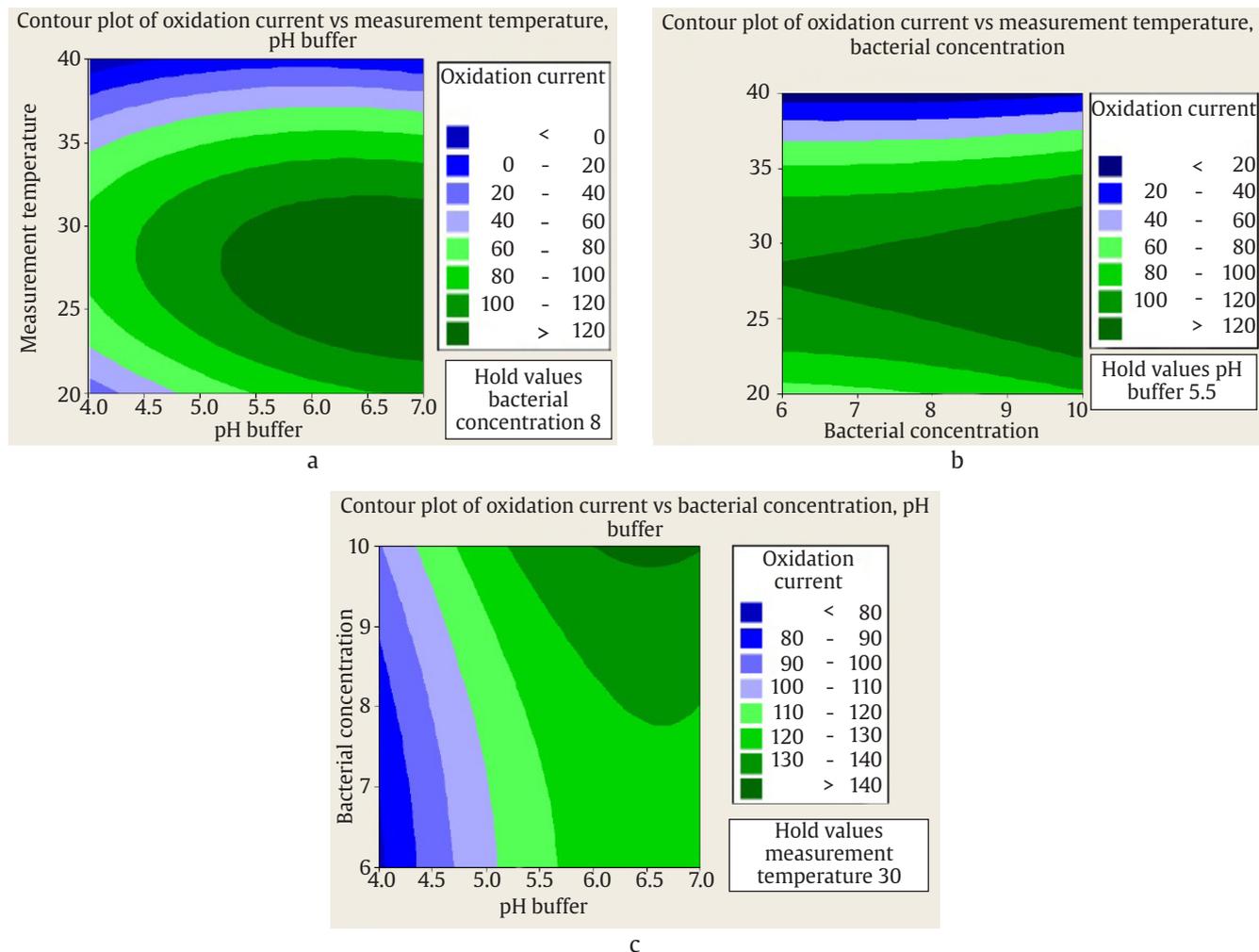


Figure 5. (a) Influence of measurement temperature and pH buffer to current, (b) influence of measurement temperature and bacterial concentrations to current, (c) influence of bacterial concentration and pH buffer to current

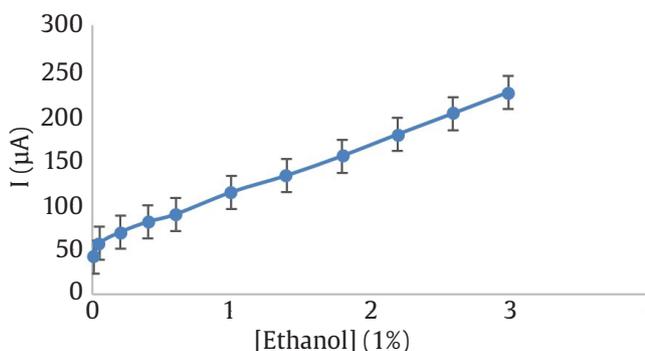


Figure 6. Linear regression curve, relationship [ethanol] with the oxidation peak current

4.2. Electrochemical Measurement

The ethanol as a substrate was detected and measured by the alcohol oxidase enzyme as the bioreceptor. Its catalytic reaction of oxidation reaction

Table 2. Coefficient of selectivity of interfering on ethanol substrate

Interfering compound	[Compound] (%)	K_{ij}
Methanol	1.5	-29.889
Methanol	1	-34.333
Methanol	0.5	-39.889
NaCl	1.5	-3.5
NaCl	1	-9.75
NaCl	0.5	-13.5
Citric Acid	1.5	-28.5
Citric Acid	1	-38.5
Citric Acid	0.5	-53.5

of ethanol to acetaldehyde with the aid of available oxygen as an oxidizer which will be reduced to hydrogen peroxide. This reverse redox reaction between oxygen and hydrogen peroxide will produce transferred electrons causing a current to be sent by electrode to transducer in the form of a signal. This signal by the

transducer is converted into electromagnetic waves which can be read and recorded by the recorder as the peak of ethanol oxidation current (Figure 3). The result of ethanol oxidation current measurements with SPCE biofilm was 122,429 μA (Figure 4). Previous studies had lower oxidation current peaks, 2,856 μA (Iswantini *et al.* 2016), 2.34 μA (Iswantini *et al.* 2017), and 750 nA (Rama *et al.* 2012). In fact the optimum pH buffer for alcohol oxidase by electrochemical method occurred at pH 6 (Rama *et al.* 2012), pH 7.2, pH 7 (Carelli *et al.* 2006; Türkarşlana *et al.* 2010).

4.3. Validation Methods of Biosensor

The biosensor's analytical parameters were evaluated based on linearity, sensitivity, limit detection, quantization limit, and precision parameters. The results showed that the ethanol concentration was directly proportional to the intensity of the oxidation peak current (Figure 6). The linear relationship between the concentration of ethanol and the intensity of the anodic peak current follows the equation $y = 57.29x + 53.03$ with $R^2 = 0.993$. Linearity is the ability of the analytical method to provide a proportional response to the concentration of the analyte in the sample. The sensitivity value obtained in this study was 57.29 μA (%) (Niculescu *et al.* 2002). The higher the sensitivity value, the more sensitive the method used. The limit of detection and quantification of the constructed biosensor were 0.003 and 0.009% respectively. These low limit values indicate that the sensitivity of the method used is very good. The obtained LD and LK values have met the ethanol concentration limit in the process of making food and beverages that was <1%. However, the obtained limit of detection was greater than those of the value of the previous research that was 0.02 mm (Rama *et al.* 2012), 0.035% (Shkotova *et al.* 2005), 2.3 μm (Carelli *et al.* 2006). Interestingly, the precision for the measurement accuracy expressed as the percent value of relative standard deviation of 1.95% indicated a good accuracy.

The kinetics analysis revealed that the biosensor had K_M value of 3.5 mm and V_{max} of 125 μA (Figure 7). The K_M value indicates the strength of an enzyme in binding to the substrate. The greater the K_M value, the weaker the enzyme binds to the substrate and vice versa. The K_M value of this biosensor was higher than that of the pure enzyme of *Hansenula* yeast which amounted to 2.4 ± 0.7 mm (Rama *et al.* 2012). However,

this K_M value was lower than that of the pure enzyme of *Pichia pastoris* yeast which amounted to 7.8 m (Türkarşlana *et al.* 2010). It seems that the difference in K_M values was due to differences in the source of the enzyme and the applied measurement methods.

The main problem of alcohol oxidase based biosensors is their limited stability (Kuswandi *et al.* 2014). The results showed that biofilm *A. aceti* on the SPCE surface remained stable after the 55th day of measurement with residual activity of 100.34%, however no measured data after that time (Figure 8). This constructed alcohol biosensor had a higher stability than that of the pure enzyme-based ethanol biosensor of *A. aceti* using a 3-day EPK with residual activity of 22.34% (Iswantini *et al.* 2016) a pure enzyme-based ethanol biosensor from *Pichia Pastoris* yeast of the 28 days with remaining activity of 20% (Türkarşlana *et al.* 2010) and 5 days (Shkotova *et al.* 2005). The ethanol biosensor of the pure enzyme from *Hansenula* yeast immobilized on SPCE reached 2 months (Rama *et al.* 2012). In terms of selectivity, the three interfering compounds with concentrations of 0.5, 1.0, 1.5 did not interfere with the performance of electrode measurements against ethanol substrates (Table 2), as

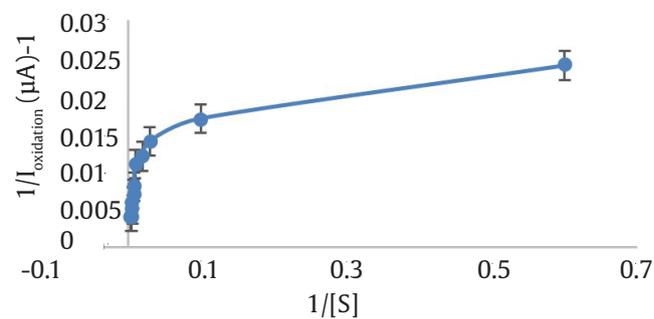


Figure 7. Lineweaver–burk curve relationship between $1/[S]$ and $1/I_{\text{oxidation}}$

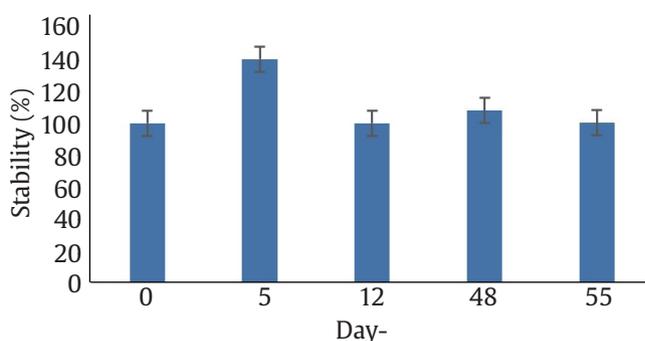


Figure 8. Stability of ethanol biosensors with SPCE biofilm *A. aceti*

indicated by the selectivity coefficient was lower than 1 (Wahab 2006). The selectivity coefficient is a factor that reduce the selectivity of electrode (Wahab 2006). Interference from other ions may affect potential readings resulting in cross sensitivity and may decrease the activity of the main ion.

5. Conclusion

The constructed alcohol biosensor of *A. aceti* biofilm on the SPCE had good and better activity and stability than those of the reported biosensor using a carbon paste electrode. The biofilm on the SPCE provide a longer viable bacterial cell producing active alcohol oxidase as bioreceptor and catalysator in the alcohol detection and measurement.

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